Profuse and selective growth *in vitro* of rat spinal axons on a micro-patterned poly (ethylene imine) grid.

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Abstract-**To distribute neurites (axons) along a surface and to guide them towards specific point targets we cultured spinal cord explants on coverslips printed with a micro-patterned grid of poly (ethylene imine) (PEI) lanes. The grid was prepared by micro-contact printing with silicone stamps. Spinal cord explants were resected from neonatal rats. A tiny bunch of glass filaments was used to ensure adhesion of the explant to the coverslip. One end of the bunch was glued to the coverslip while the other end pressed the explant firmly onto the coverslip. Spinal cord explants cultured in a collagen matrix, on a coverslip stamped with poly D-lysine (PDL), or on a coverslip uniformly coated with PEI or PDL were used as controls. Cultures were maintained for 6 days *in vitro* (div). Outgrowth from the explant was observed using phase contrast microscopy.**

None of the explants detached from the coverslip. Neurites emerged randomly from the explants, but upon crossing one of the grid lanes they subsequently followed the grid pattern. The outgrowing neurites were guided by the PEI lanes of the grid and reached lengths of up to 2400 µm.

After 6 div no signs of degeneration were observed in the outgrowth of explants cultured on a stamped coverslip or on a homogeneously coated coverslip, while degeneration did appear in the explants cultured in collagen after 4 div.

Compared to control explants cultured on PDL (either on stamped or on uniformly coated coverslips), explants cultured on a micro-patterned PEI grid grew more profuse (more and longer neurites).

Because these cultures can be easily manipulated, this paradigm is ideally suitable for studies of neuronal networks and for studies that necessitate the guidance of neurites towards a specific target (in culture), for instance the electrodes of a multi-electrode array in a culture dish.

Keywords - **culture; neonate; outgrowth; rat; spinal cord; stamp; print; PEI; multi-electrode array**

I. INTRODUCTION

The mechanisms of growth cone orientation are extensively investigated, but poorly understood. Growth cones, and the neurites that are attached to them can be experimentally guided by diffusible cues, by the means of an electric field, by nerve guides (mechanical constraints), or by artificial paths.

Precise guidance of neurites would be a major advantage for many experiments. It would allow, for instance, for the construction of artificial networks with real neurons. It would also permit for the connection between spinal cord neurons *in situ* and a cultured multi-electrode array (MEA). Such arrays, conceived as interfaces between electronic devices and the nervous system, will consist of an implantable encased plate containing electrodes covered with clusters of cultured neurons. Those cultured neurons will be connected to the central nervous system through (induced) collaterals of peripheral nerves [1]. This technique aims to support, and eventually to restore motor function after spinal paralysis.

Micro-patterned grids of adhesive substrates (used alone or in combination with other techniques) may be used to precisely target neurites toward specific points. This technique was successfully applied to dissociated neurons from chicken, which grew on poly D-lysine (PDL) lanes.

Rats are useful placental mammals for the study of regeneration. As in humans, the development of their nervous system is not complete at birth. Recent studies show that successful regeneration of outgrowth can still be obtained *in vitro* with neonatal rats [2]. Interestingly this method (culturing explants in collagen) yields long outgrowth (2 mm in 2 div).

For the present study, a model was conceived combining rat spinal explants (in order to have profuse outgrowth), and micro-patterned printing of adhesive substrate for guidance of the neurites. Previous screening of potential adhesive substrates suggested that poly (ethylene imine) (PEI) would be better than PDL for this model.

Dissociated cells or small pieces of tissue adhere easily to a substrate, but the larger an explant is, the most susceptible it is to detachment, due to eddies in the surrounding culture medium. Therefore a new method was conceived to get the explant to adhere to the substrate. Two techniques are generally used to secure an explant to the substrate. The explant is deposed onto the substrate within the smallest drop of medium, and then submitted to evaporation. The explant is pressed onto the substrate by its own weight. When it is assumed that the explant adheres sufficiently, but before it starts to dry out, the Petri dish is filled up with culture medium. Progressive concentration of the medium during the evaporation phase might be toxic for the explant and variable between explants (precluding comparative studies). With another method the explant is secured onto the substrate with a dialysis membrane; after a couple of hours, the membrane is removed. In both cases it is difficult to assure that the explant will remain adhered.

When culturing a large explant on a micro-patterned grid, the problem is even larger as most of the culture surface will not be covered with the adhesive substrate. For this reason a new technique allowing for solid adhesion of the explant to the support was needed. The explant is secured on the coverslip with a few glass

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Abstract

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filaments assuring immobilization of the explant and free diffusion of the medium around the explant. Using this technique we obtained profuse outgrowth (as compared to explants cultured on a PDL grid), and relatively long-term survival of the explants (as compared to explants cultured in a collagen matrix). Moreover, the outgrowing neurites were guided by the microprinted lanes. Results that we obtained with this method were briefly mentioned [3], but the method was not extensively described.

II. MATERIALS AND METHODS

We used stamps to print a pattern on glass and we used glass filaments to secure the explants on the glass. We cultured those explants for 6 div in a chemically defined serum free medium R_{12} [4] and we observed the outgrowth with phase-contrast microscopy.

The procedure for making the stamps has been extensively described in 1999 by Warbinek [5]. In short, the pattern was designed on computer and then engraved in glass using micro-techniques (photolithography, dry-etching and wet-etching). The grooves in the resulting mold are 25 µm deep and 75 µm large. Silicon was poured on the resulting mold, solidified in an oven and turned out. This silicon block was used as the micro-stamp.

PEI is transferred from the stamp to the coverslips by contact. We know from experience that the choice and the treatment of the glassware are of major importance for the quality of the growth (length and number of the neurites) and for its specificity (the tendency to be guided by the printed lanes). Coverslips 18 x 18 mm (Menzel-Gläser, Germany) were immersed for 1 week in potassium dichromate 10 %, rinsed till no more coloration is visible, rinsed 2 more times in distilled water, plunged in ethanol 70 % (to change surface properties) and allowed to dry. Using a fine diamond pen a cross was drawn in the middle of the coverslip on the future underside and an asymmetric mark was drawn in one corner. These signs were used for positioning of the stamp and the explant and to differentiate the 2 sides of the coverslips. Thereafter the coverslips were sterilized (4 hrs, 180ºC).

The stamps were made hydrophilic by argon plasma cleaning (Biorad sputter coater, 3 min. at 2.5 kV), and stored. After every 10 stampings the stamp must be made hydrophilic again. Immediately prior to printing the stamp was wetted with PEI (0.2 mg/ml; Sigma Aldrich Chemie, Steinheim, Switzerland) for 1 min, then dried with filtered pulsed air. The stamp was then positioned onto the coverslip and manual pressure was applied. Equal application of pressure was observed with an inverted microscope, allowing illumination and observation from below, of the to-be-printed coverslip. Pressure was maintained for 1 min. Transferred PEI could be observed by transillumination with an inverted microscope at high magnification.

All the coverslips were laid down on paper. A bunch of a few glass filaments (10 mm long) was deposited on each coverslip. The filaments were dispersed by gently rolling the tip of a pair of curved tweezers over them.

A collagen solution was prepared extemporaneously from:

- 800 µl of Vitrogen®, (3 mg/ml bovine type I collagen solution; Collagen Corporation, Fremont, CA)
- 100 µl of a solution containing 38 mg/ml $NaHCO_3$ and 135 mg/ml DMEM (ICN Biomedicals, Zoetermeer, NL)
- 100 µl of 0.1N NaOH
- 15 µl 1 N HCl

The collagen solution was stored in melting ice.

One end of the bunch was fixed in place with a drop of the collagen solution (used as glue). In order to immobilize the filaments the end is held with the tip of a pair of curved tweezers and the drop of collagen is deposed onto it. The coverslip was placed into a Petri dish (Figure 1) and was stored immediately in a CO_2 incubator (37°C, 5.5 % CO_2). After 2 hrs (time necessary for the gelation of the collagen) 1 ml of culture medium (R_{12}) was added to each Petri dish.

As controls, spinal cord explants were cultured in a 3-D collagen matrix [6] or cultured on coverslips entirely coated with PEI, printed with PDL (Sigma Chemical Co, MO) or entirely coated with PDL.

All procedures for the preparation of spinal explants took place under antiseptic conditions and in accordance with national laws. The spinal cord from new-born Wistar albino Glaxo rats was collected in R_{12} medium and the meningial covering was stripped away. The lumbar enlargement was chopped immediately into 250 µm slices. These slices were separated into lateral halves. Five of the resulting slices were selected and immediately distributed into the culture chambers. The glass filaments were lifted with a fine blade and the explant was inserted under them. The five cultures are placed in a glass dish, and gently rotated, to ascertain that all explants are indeed held firmly down by the glass fibers. If necessary, explants were replaced under the glass filaments or in a new culture chamber. Cultures are then placed back into the incubator for 6 div. The culture medium was refreshed every other day. Observations and photomicrographs were made using phase contrast microscopy.

III. RESULTS

Despite the manipulations (transportation, observation and refreshment of the medium) none of the explants detached from the Petri dish (n = 74) after the beginning of the culture.

Explants cultured on PEI lanes displayed long and profuse outgrowth. Neurites emerged randomly from the explants, but upon crossing one of the grid lanes they subsequently followed the grid pattern (Figure 2). Neurite bundles were segregated along the side of the grid lanes, presumably growing along the most concentrated sites of PEI deposition. After 6 div the bundles of axons reached lengths of up to 2400 µm. The outgrowing neuritic bundles tended to distribute

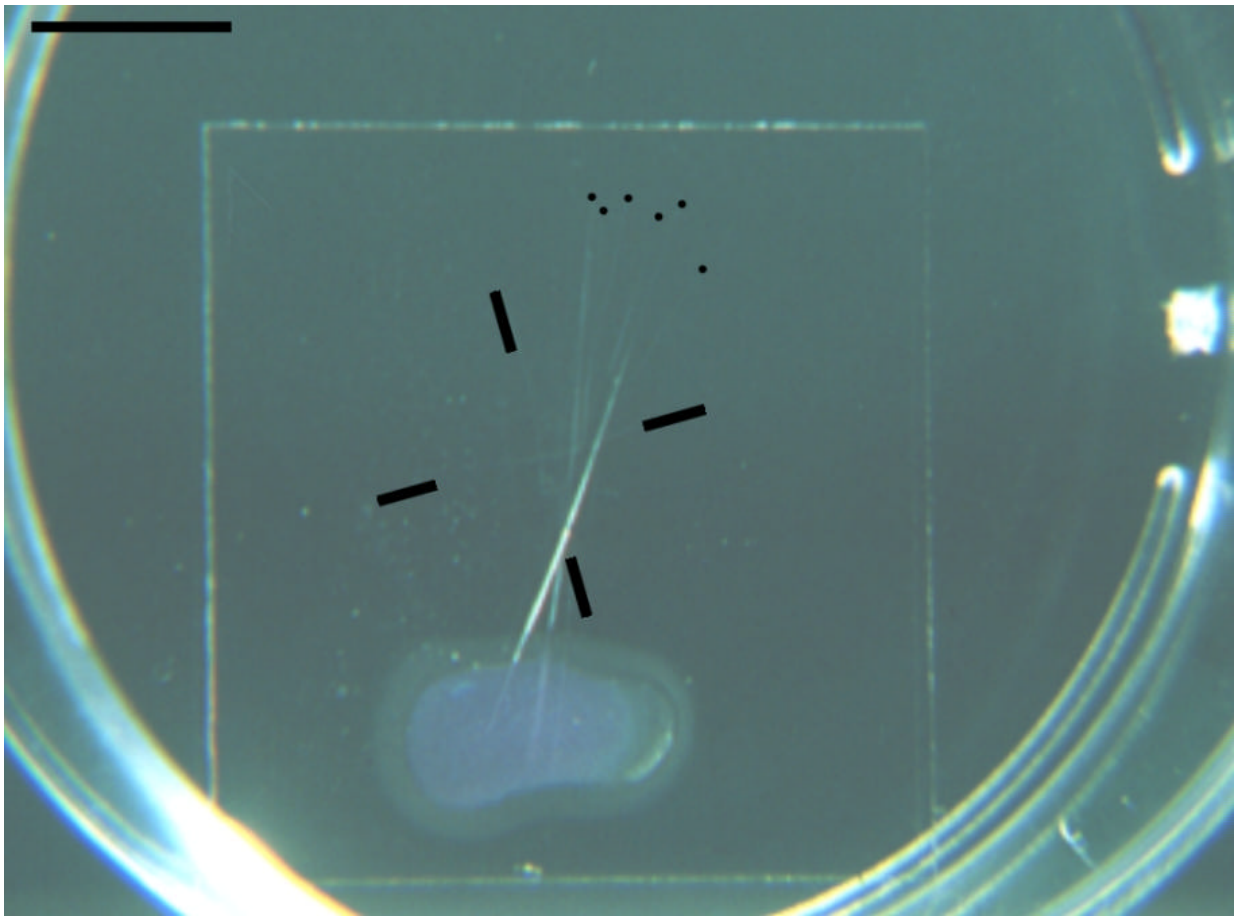

Figure 1
Representative photograph of the culture chamber. The outmost round structure is the wall of the Petri dish. The square structure is the coverslip. The round structure on the coverslip is the gelled drop of collagen. The cross drawn on the opposite face of the coverslip has been pointed out with 4 dashes. The vertical structures are 6 glass filaments; the lower ends are immobilized within the collagen drop, the upper ends free (pointed out with 6 dots). The asymmetric mark is seen in the upper left corner of the coverslip. Bar is 5 mm.

themselves over the grid pattern, by branching or by subdivision into smaller bundles at the intersections, progressively occupying the available grid lanes while growing outward from the explant.

As predicted [5] the printed pattern of PDL (control) is not visible (The authors monitored the amount of PDL present on the substrate by previously labeling the PDL with fluorescein isothiocyanate). Upon printing with PEI a vague pattern can be seen by direct microscopic observation.

Culture on PEI (lanes or coating) yielded far more profuse and much longer outgrowth than control culture on PDL (lanes or coating). Cultures on lanes (PEI or PDL) yielded patterned outgrowth while other controls didn't. Control explants cultured in a collagen matrix displayed similar profuse outgrowth as cultures on PEI but the first signs of degeneration could already be seen after 4 div (Figure 3).

The neurites grew specifically on the grid lanes. Few neurites could be seen that crossed the non-covered glass surface between the lanes. This indicates a far higher probability for the neurites to grow on the PEI lane that on non-covered glass. In the vicinity of the explants some neurites can be seen that cross between lanes possibly because the density of axons is much higher here. Some of these have a linear structure indicating that they are not adhering to the glass but are stretched upon it (Figure 2).

No signs of degeneration appeared during the time of the experiments. Experiments investigating the maximal possible length of culture (data not shown) indicated that first signs of degeneration would appear only after 1 week in culture; the presence of non-growing neurites without growth cone, the appearance of nodules along the shaft of the neurites. Eventually the neurites would die. They change itself into lanes of debris that will eventually disappear.

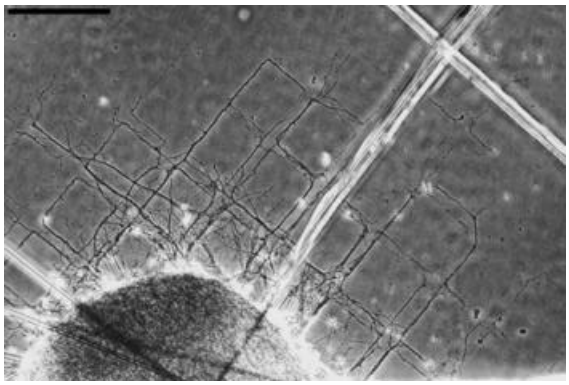

Figure 2
Outgrowth from a neonatal rat lumbar spinal cord explant, after 6 div. The oblique linear structures in the lower left corner are three glass filaments. The two perpendicular lines that cross each other in the upper right corner are the scratches made with the diamond pen. Phase contrast microscopy, bar 500 mm.

IV. DISCUSSION

No explants detached from the coverslips. As far as we know our culture chambers are the best way to attain firm adhesion of an explant to the substrate. This technique is simple, it also permits to start the culture in a very short time after resecting the explants and it permits easy access to the outgrowth, enabling easy manipulation of the neurites. The technique is based on the properties of glass (inert, strong and sterilizable) and of collagen (sticky, innocuous and sterile).

The longer time during which the culture is free of degenerative signs, as compared to control culture in collagen might be explained by better diffusion of the medium around the explant.

The length of the outgrowth, the long culture period, and the specificity of the growth on the printed pattern make this method ideal for future studies.

Axons could be specifically directed toward the neuronal clusters cultured on the microelectrodes of a cultured probe. A growth cone could be forced to orient itself toward a specified lane (by using electrical fields, diffusible tropic factors, or by blocking a lane with a scratch or with repulsive factors). Future experiments will determine whether the PEI grid persists sufficiently long for application *in vivo*, as there will necessarily be a substantial delay between the initiation of axons collaterals from the nerve and the moment when the growth cone will synapse to the clusters of the MEA.

Incidentally the same experimental paradigm (explant culture on patterned substrates on MEA) is ideally suitable for the study of collateral induction through electrical stimulation [7]. These studies are currently in progress.

Finally explants cultured on superimposed patterns of different adhesive substances may be employed to obtain even more intricate and coordinated networks of axons *in vitro*. In this regard the relative poor outgrowth from the spinal explant on PDL lanes is an interesting property. A protocol might be created in which spinal cord explants would develop outgrowth preferentially on PEI while another type of nervous tissue would grow preferentially on PDL.

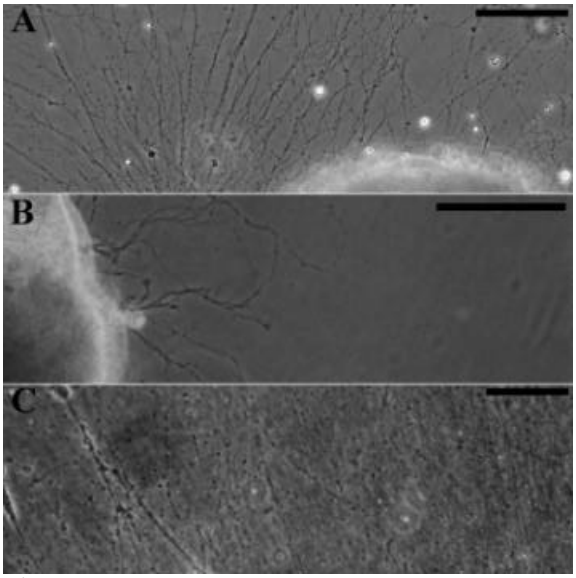

Figure 3
Representative photographs of controls. Phase contrast microscopy, bar 100 µm. A. Profuse and long outgrowth on glass entirely coated with PEI, after 6 div. B. Outgrowth on glass entirely coated with PDL, after 6 div. Neurites are shorter and less numerous. C. Profuse and long outgrowth in a 3D collagen matrix, after 4 div. First signs of degeneration (nodules, debris in line) are present.

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